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VOLATILE CHEMICAL CUE ELICITS MATING BEHAVIOR OF COHABITING MALES OF *NEPHILA CLAVATA* (ARANEAE, TETRAGNATHIDAE)

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ABSTRACT. Previous studies have revealed that matings of the spider *Nephila clavata* are concentrated just after the final molt of females. We determined the existence of a chemical cue eliciting male mating behavior in this particular period by three experiments. First, an immobile spider anesthetized with CO_2 was placed in a web, and the behavioral response of an introduced male was observed. Males approached and exhibited mating behavior to newly-molted immobile spiders irrespective of the stage and sex, while these behaviors seldom occurred to immobile spiders that were more than 24 hours past molting. Secondly, an olfactometer experiment revealed that newly-molted females attracted males much more frequently than females more than 24 hours past molting. Thirdly, the acetone-soluble extract of the body surface of newly-molted females was absorbed on to a piece of filter paper, and it was placed near the female which had been killed. Males approached and touched the female having the extract more frequently than the female without it. These results indicate the existence of a volatile chemical cue emitted by newly-molted individuals which elicits mating behavior in cohabiting males. This cue may be a compound involved in the molting fluid and may not be a special substance designed for sexual communication.

Adult males of the orb web spiders in the genus *Nephila* cohabit with females on their webs, and they copulate just after the final molt of females or while the females are feeding on large prey (Robinson & Robinson 1973; Christenson et al. 1985; Miyashita 1993). In *N. clavata*, most matings occurred just after the final molt of the females (Miyashita 1993). Since this species shows no obvious courtship behavior (Yoshikura 1987) and females are much larger than males, females are dangerous partners for males. However, safe copulation should be ensured when males copulate with newly-molted females which have soft exoskeletons and are not aggressive toward males. Moreover, Christenson & Cohn (1988) have demonstrated that *N. clavipes* has first male sperm precedence. This enhances the importance of early copulation and may have driven the evolution of precopulatory guarding by males. Preliminary observations have indicated that a male introduced artificially onto the web of a newly-molted female will approach the female immediately and copulate. This suggests that a chemical substance emitted by newly-molted females may elicit mating behavior of cohabiting males.

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There have been many lines of evidence for the existence of sex-related chemical cues in spiders; some are air-borne (Blanke 1975; Tietjen 1979; Olive 1982; Watson 1986; Schulz & Toft 1993) and others are contact pheromones that are borne by web silk (Ross & Smith 1979; Tietjen & Rovner 1982; Suter & Renkes 1982; Jackson 1986; Suter & Hirscheimer 1986). However, there has been no evidence that volatile chemicals emitted by newly-molted females induce male mating behavior.

The aims of the present paper are to test for the existence of a chemical emitted by newly-molted females and to determine whether this substance is unique to newly-molted adult females.

METHODS

Reproductive biology of the spider.—*Nephila clavata* has one generation per year. After overwintering as eggs, spiderlings emerge in June in central Japan. Males begin to reach maturity from late August while females mature in September and early October. Adult females are much larger than adult males (females, 12–28 mm body length; males, 3–9 mm). Adult males do not construct their own webs but cohabit on the webs with females. When more than one male attends a female, male-male combat is occasionally observed. The larger male usually occupies the hub position on the web and has the advantage in mating. More than 80% of copulations were observed just after the final molt of females (Miyashita 1993). Oviposition takes place from mid-October to November, and only one clutch is laid by a female.

Subjects and housing.—All individuals used in our experiments were collected from small woodlands in Tokyo and Yokohama City in Japan. Adult males were collected from female webs in late August and early September when no adult female was present. Each male was kept in a small plastic cup (*ca.* 10 cm × 4 cm) and was supplied with water by spraying every two days. All males in the experiments were used within two weeks following capture. Subadult males collected in September were reared individually in a cage that was 45 cm × 45cm × 45 cm. The top and the bottom of the cage were made of wooden plates, two sides were of fine mesh net, and the other two sides were sheet vinyl. A mealworm was supplied to each spider every 2–3 days. We reared spiders at room temperatures and under a natural light cycle.

Females that are near to molt are easily identified in the field by their characteristic abdominal coloration and by their reduced spiral threads. To obtain newly-molted individuals, females were collected in September and were housed as described above. Most females molted within a few days of collection. Females that were more than 24 hours past molting were collected from the field in September and early October, and the developmental stage (adult or subadult) was determined by inspecting the epigynum.

Responses to whole spiders.—In order to determine whether only newly-molted adult females elicit mating behavior of males, we examined the response of males to six groups of spiders: 1) newly-molted (1–3 hours after molting) subadult females (NSF), 2) subadult females more than 24 hours after molting (SF), 3) newly-molted adult females (NAF), 4) adult females more than 24 hours after molting (AF), 5) newly-molted adult males (NAM), and 6) adult males more than 24 hours after molting (AM).

The experimental procedure was as follows. First, we introduced a SF spider into the cage described above to obtain an intact web in the cage. After construction of the web, the spider was removed from the cage. Another spider that belonged to one of these six groups was anesthetized with CO_2 for about an hour. This immobile spider was placed gently at the center of the web with forceps. The spider hung head-up on the upper side of the inclined orb-web. Lastly, we introduced an adult male onto the lower part of the web (about 40 cm below the immobile spider), and the subsequent behavioral response of the male spider to the immobile spider was recorded. We recorded the following five behavioral traits of the male. 1) "orientation": the male spider walked toward the immobile spider and the distance between them was less than 3 cm; 2) "contact with legs"; 3) "contact with palps": the male spider touched the cephalothorax or abdomen of the immobile spider by palps; 4) "repeated contact": the male spider continued "contact with legs" for 10 minutes or it repeated "contact with palps" three times. Actual copulations were not observed, probably due to the unnatural posture and/or anesthetization of the immobile spider. Each experiment was terminated when either of the following three conditions occurred: 1) the male spider went out of the web, 2) "repeated contact" was recorded, or 3) the male spider remained motionless for 30 min. This experiment was conducted under windless conditions.

Olfactometer.—We used an olfactometer for examining whether male spiders approach females by means of air-borne substance (Fig. 1). Wire-net was attached to the inner side of the walk tunnel so that male spiders could walk easily. The female box and the walk tunnel were separated by a double fine-mesh wire-net, which made the female invisible to the male. The air current in the olfactometer was created by a fan attached to the ventilation. The wind speed was set at 11 cm/sec, which did not seem to prevent spiders from walking upwind. We first introduced a female into the female box (either NSF, SF, NAF, or AF). Next, a male was introduced into the entrance of the walk tunnel. Two hours later, we checked the location of the male, and if it was located within 15 cm from the double wire-net separating the female box and the walk tunnel, it was regarded as a positive response. At the end of each experiment, the olfactometer was washed with acetone. Experiments with no individuals in the female box were conducted as controls.

Responses to extracts.—We examined whether an extract of the body surface of newly-molted females elicited mating behavior in males. The experimental procedure was as follows. (1) Sub-

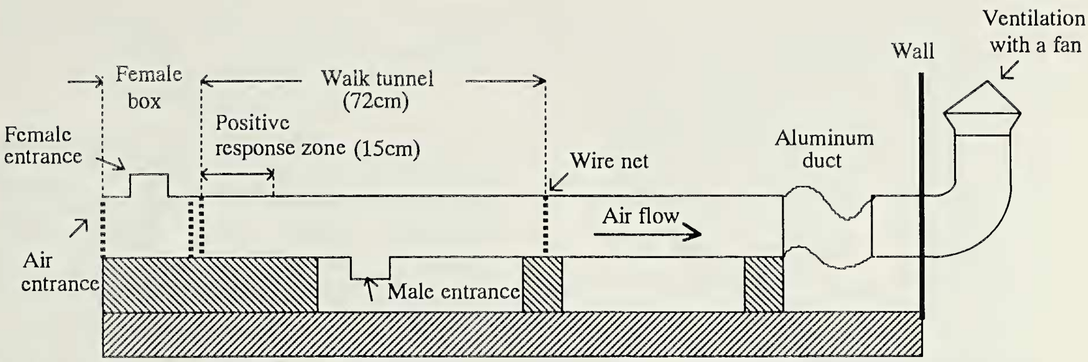

Figure 1.—The olfactormeter used in Experiment II. "Walk tunnel" is made of a transparent acrylic pipe.

adult females that were more than 24 hours past molting were killed in a refrigerator, to be used as "dummy females". (2) A total of 12 newly-molted adult females was killed with CO_2 and then soaked in 15 ml of acetone for five minutes. This fluid may contain chemical substances derived from the body surface as well as from the internal organs, and is called the extract. Since the fluid remained clear after spiders were soaked, it probably contained little material from the internal organs. (3) An adult female, more than 24 hours after molting, was released into the cage and then removed after completion of the web. (4) A "dummy female" was placed at the center of the web as the same manner as in Experiment I. (5) We let fall a few drops of extract (concentration: about 0.2 female equivalent) or acetone (control) on a small piece of a filter paper (1 cm × 1 cm) and dried the paper in air for a few minutes. It was then carefully placed on the web three cm below the "dummy female". (6) Finally, an adult male was introduced onto the web and subsequent behaviors were recorded as in Experiment I. The experiment was conducted under windless conditions.

RESULTS

Responses to whole spiders.—Responses of males were greater to newly-molted individuals than to those more than 24 hours past molting. This relationship was observed consistently for subadult females, adult females, and even adult males (Table 1). The behavioral traits most characteristic of mating were "contact with palps" and "repeated contact"; and these two behaviors were seldom performed toward individuals that were more than 24 hours past molting, irrespective of the stage and sex. On the other hand, nearly half of the males touched the newly-molted individuals with their palps and showed "repeated contact" (Table 1).

For newly-molted individuals, there were no significant differences between subadult and adult females in all behavioral traits of males (Table 1); and, also, there were no differences in male responses to adult males and females (Table 1). Similarly, for individuals more than 24 hours past molting, there were also no significant differences between ages nor between sexes. These results strongly suggest that newly-molted individuals, irrespective of the stage and sex, produce the stimulus attracting and causing mating behavior in males.

Olfactometer.—In the control experiment in which no female was present in the "female box", 17% of males were located in the area of "positive response" (Table 2). This may be regarded as the result of random walking within the tunnel. Significantly larger percentages of males exposed to newly-molted females were located in the positive response area than those in the control (Table 2). On the other hand, no differences were found in the frequencies of males showing positive response between the control and those exposed to females more than 24 hours past molting (Table 2). These results are consistent with those of the experiment with whole spiders and show that an air-borne substance emitted by newly-molted females attracted the males.

Responses to extracts.—All males showed "orientation" and 90% of them exhibited "contact with legs" to "dummy females" with extract, while 20% of males showed the above behaviors to the females with acetone (control, Table 3). Thus, body surface extract of newly-molted females attracted males. However, there were no differences between the controls and the females with extract in the frequences of males showing

Table 1.—Number of *Nephila clavata* males that exhibited each behavioral response to anesthetized immobile conspecific spiders on webs in Experiment I. Values in parentheses represent percentages of the total numbers. Differences in frequencies between immobile spiders were tested by Fisher's exact probability test. Asterisks attached to probabilities indicate that these probabilities are significant at 5% level of adjusted significance ($\alpha = 0.0073$; Dunn-Sidák method) with seven pairwise comparisons. NSF: newly molted subadult female, SF: subadult female, NAF: newly molted adult female, AF: adult female, NAM: newly molted adult male, AM: adult male.

Male behavior	NSF		SF		NSF vs. SF	NAF		AF		NAF vs. AF	NAM		AM		NAM vs. AM	NSF vs. NAF	NAF vs. NAM	SF vs. AF	AF vs. AM
	+	−	+	−	(*P*)	+	−	+	−	(*P*)	+	−	+	−	(*P*)	(*P*)	(*P*)	(*P*)	(*P*)
Orientation	9 (90)	1 (10)	7 (23)	23 (77)	<0.001*	17 (71)	7 (29)	5 (25)	15 (75)	0.003*	5 (71)	2 (29)	6 (21)	22 (79)	0.021	0.231	0.681	0.685	0.740
Contact with legs	9 (90)	1 (10)	4 (13)	26 (87)	<0.001*	14 (58)	10 (42)	4 (20)	16 (80)	0.011	5 (71)	2 (29)	4 (14)	24 (86)	0.006*	0.077	0.435	0.401	0.442
Contact with palps	6 (60)	4 (40)	1 (3)	29 (97)	<0.001*	11 (46)	13 (54)	0 (0)	20 (100)	<0.001*	4 (57)	3 (43)	2 (7)	26 (93)	0.009	0.354	0.461	0.600	0.335
Repeated contact	6 (60)	4 (40)	0 (0)	30 (100)	<0.001*	11 (46)	13 (54)	0 (0)	20 (100)	<0.001*	3 (43)	4 (57)	1 (4)	27 (96)	0.019	0.354	0.617	1.000	0.583

"contact with palps" and "repeated contact" to the females (Table 3).

DISCUSSION

Evidence of a volatile chemical cue.—Male spiders approached newly-molted individuals and exhibited mating behavior such as touching them with palps, but they rarely showed such responses to individuals that were more than 24 hours past molting (Table 1). The webs used in the whole spider experiment were all derived from subadult females that were more than 24 hours past molting. Moreover, anesthetized immobile spiders could not add silk to the webs. Therefore, it seems that a volatile substance from the body, and not from the silk, elicited orientation and mating behavior of males. This was strongly supported by the olfactometer experiment in which males were attracted upwind toward newly-molted, invisible females (Table 2).

The frequencies of males trying "contact with palps" and "repeated contact" were not very high (about 50%) in the experiment with whole spiders. This could be due to the unnatural posture of the immobile spider, which hung head-up on the upper side of the inclined orb-web. Alternatively, immobilization of spiders by CO_2 itself may prevent normal mating behavior of males. In the olfactometer experiment, the percentage of males that approached newly-molted females was 60% (Table 2). This may indicate that the attractiveness of this volatile substance is not

Table 2.—Number of *Nephila clavata* males located at the positive response zone within the olfactometer in Experiment II. Values in parentheses are percentages of total males. Differences in frequencies between treatments and a control were tested by Fisher's exact probability test. Asterisks attached to probabilities indicate that these probabilities are significant at 5% level of adjusted significance ($\alpha = 0.013$; Dunn-Sidák method) with four pairwise comparisons. NSF: newly molted subadult female, SF: subadult female, NAF: newly molted adult female, AF: adult female, control: no spider.

Spiders tested	Response +	Response −	Comparison with control (*P*)
NSF	6 (60)	4 (40)	0.019
SF	2 (11)	16 (89)	0.481
NAF	15 (60)	10 (40)	0.002*
AF	3 (17)	15 (83)	0.665
Control	4 (17)	20 (83)	

Table 3.—Number of *Nephila clavata* males exhibiting each behavioral response to dummy females with or without extract of the body surface of newly molted adult females *N. clavata* (Experiment III).

Male behavior	With extract +	With extract −	Without extract +	Without extract −	Fisher's test *P*
Orientation	10 (100)	0 (0)	2 (20)	8 (80)	<0.001
Contact with legs	9 (90)	1 (10)	2 (20)	8 (80)	<0.001
Contact with palps	1 (10)	9 (90)	0 (0)	10 (100)	0.500
Repeated contact	1 (10)	9 (90)	0 (0)	10 (100)	0.500

very strong over long distances. Nevertheless, to attract and cause mating behavior of males cohabiting in the same web, this substance should be effective because the distance between the two sexes in nature is, at most, about 30 cm (pers. obs.). Other mechanisms must be used by males to locate female webs.

The experiment with extracts revealed that a chemical substance present on the body surface of newly-molted females attracts males (Table 3). However, males rarely touched the dummy female with their palps, which is a characteristic mating behavior. This is probably because extract from the female body surface is on the small filter paper placed near the dummy female, not on the body of the dummy female. Although males are attracted to females by the volatile substance and touched the body with their legs, subsequent mating behavior may require chemical stimulus that is detected when the legs contact the body surface of newly-molted females.

Characteristics of the chemical cue.—The chemical cue found here is an air-borne substance emitted by newly-molted individuals which causes mating behavior of cohabiting males. It is especially noteworthy that not only do adult females produce this cue but subadult and even adult males do, also.

There have been several reports on the existence of air-borne sex-related chemical cues in spiders (Blanke 1975; Tietjen 1979; Olive 1982; Watson 1986; Schulz & Toft 1993). All of these spiders use chemical cues to attract males from outside their webs, but they do not appear to use it to elicit mating behavior of cohabiting males. Chemical cues causing orientation and courtship behavior of cohabiting males (i.e., within the web) are known in some spiders (Ross & Smith 1979; Suter & Renkes 1982; Suter & Hirscheimer 1986), but all of these are contact cues contained in the web silk. Thus, the sex-related chemical cue of *N. clavata* has a unique characteristic: although it is air-borne, it elicits mating behavior of cohabiting males, which is functionally similar to the contact cues mentioned above. To our knowledge, this type of chemical cue has not been published previously.

Another important feature of this cue is that it is emitted just after the molt. In a jumping spider *Phidippus johnsoni*, the contact pheromone that arrests males near the immature female is contained in the web silk of females which are about to molt (Jackson 1986). However, this functions to make males cohabit with females, not to stimulate mating behavior. In other Arthropoda, there are a few examples of sex pheromones which are emitted at or near the time of the molt: males of some crab species exhibited a typical display behavior when exposed to the water from a tank containing a premolt female crab (Ryan 1966; Kittredge & Takahashi 1972); and males of some butterflies gather around pupae that are about to eclose (Brown 1981; Elgar & Pierce 1988). Elgar & Pierce (1988) have suggested that a volatile substance emitted by late-stage pupae attracts males of the Lycaenid butterfly, *Jalmenus evagoras*, because if an observer crushes a late-stage pupa, the person's fingers also become attractive to males. Interestingly, this butterfly cannot detect the difference between male and female pupae, which is similar to *N. clavata* because males exhibited mating behavior to both newly-molted males and newly-molted females. Therefore, the chemical cue of *N. clavata* seems to have an origin similar to that in the crabs and the butterfly described above: all of them must be derived from some compounds related to molting. The chemical cue of this spider may be a compound involved in the molting fluid, and is probably not a substance specially designed for sexual communication because it is hard to imagine that males and juveniles developed a special machinery with no benefit to them. In this context, the cue found in the present study is neither a communicating substance nor a sex pheromone in the strict sense (Krebs & Davies 1993; Williams 1992).

Significance of the chemical cue.—In spiders, males are usually smaller than females. This is especially true for *N. clavata* in which male body length is ½–⅓ of female body length (Miyashita 1993). Moreover, males of this species show no obvious courtship behavior to females (Yoshikura 1987), unlike many other orb-weavers (Robinson & Robinson 1980). Thus, the predatory nature of the spider makes females potentially dangerous for males. Just after the final molt of a female, however, a male is able to copulate without the danger of being eaten by a large partner because she is not aggressive due to her soft exoskeleton. Furthermore, selection must have favored early copulation since first male sperm precedence is known in a congener *N. clavipes* (Christenson & Cohn 1988). Thus, the adaptive significance of males using an odor emitted by just molted females is obvious.

Since males approached and exhibited mating behavior to males and subadult females which had just molted, one may consider the disadvantage of using such a non-specific cue. Despite intensive field observations conducted for three years, no adult males were found to cohabit on subadult male webs (Miyashita pers. obs.). Thus, attempting to mate with a newly-molted male is unlikely in nature because the chemical cue seems to be effective only within the web. We observed two instances in which a male was trying to copulate with a newly-molted subadult female. Also, Christenson et al. (1985) reported two examples of juvenile copulation in *N. clavipes*. This seems rather exceptional in nature, however, because the probability of an adult male cohabiting with a female of a pre-subadult stage is low. Therefore, the disadvantages of using a non-sex-specific and non-stage-specific cue appears to be small, and hence selection has favored males that responded to a chemical cue without a sexual function that was produced by newly-molted individuals. A similar view of the origin of some sex pheromones was proposed by Kittredge & Takahashi (1972) and Thornhill (1979).

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